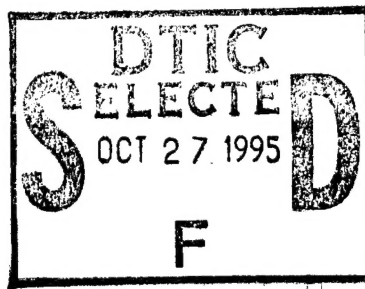


Hawaii Biotechnology Group, Inc.
1 January 94 - 31 December 94



N00014-93-C-0019
Second Year Report

I. Overview

The following report covers progress made during the eighth quarter (10/1/94-12/31/94) as well as that for the entire second year (1/1/94-12/31/94) of contract number N00014-93-C-0019. Originally, the contract was to end during December 1994; however, due to a temporary suspension of funds and subsequent reductions in work effort, the work was not completed. As adequate funds are still available, we have requested a no-cost extension. For this reason, we are submitting a second year report in lieu of a final report at this time.

The four topics that comprise this contract and their current statuses are as follows:

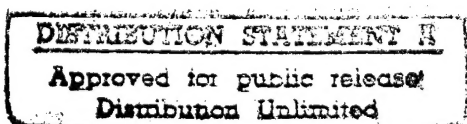
1. The production of anti-ferret IgA antibodies. Status: **Immunization of rabbits and collection of sera completed. Mouse ascites successfully cross-adsorbed; material sent to NMRI researchers in 12/94.**
2. The purification of lipopolysaccharide from *Shigella* and *Campylobacter* species. Status: *Shigella sonnei* and *S. flexneri* LPS delivered to NMRI researchers. Purification of *C. jejuni* LPS reinstated. Have collected 173mg of required 200mg LPS. Production of remainder in progress.
3. The development of an enzyme immunoassay for the detection of enteroaggregative *Escherichia coli* heat-stable toxin. Status: **Initial recombinants provided by Navy unsatisfactory. Work suspended in 10/93 due to contract funding reduction. Suitable recombinants still not available.**
4. The production of monoclonal antibodies against strain-specific antigenic epitopes on *Campylobacter coli* flagella. Status: **Completed 3 fusions. No strain specific MAb identified.**

II. Current Progress

1. Production of Anti-ferret IgA Antibodies

Immunoglobulin A (IgA) is the predominant immunoglobulin present in secretions such as milk and saliva and may be the first specific defense against natural infection. In serum, the molecule is present as a monomer; whereas, in secretions, IgA is present predominantly as dimers, although trimers, tetramers, and pentamers also exist. When in the dimeric form, the IgA monomers are covalently linked via intermolecular disulfide bonds between the heavy chain constant regions of the IgA monomers. Polymerization of IgA is initiated by a 14 kilodalton

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PROGRESS REPORT
N00014-93-C-0019
TITLE: HAWAII BIOTECHNOLOGY
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protein, designated "J-chain", which binds to the C-terminal cysteine of one of the monomers. In addition, a 70 kilodalton protein known as "secretory component" is linked to the macromolecule by either covalent or non-covalent forces; its function is to protect the molecule from proteolytic degradation.

Secretory IgA (sIgA) is thought to play a significant role in the prevention of diseases of the mucosal epithelia, such as bacterial diarrhea. Thus, studies on the production of sIgA by the host in response to the invading pathogen can provide valuable information regarding the disease process. For these studies, the use of an *in vivo* model which mimics the disease process in humans is of utmost importance. For the study of campylobacter infection, ferrets are currently used as a model. To facilitate study of the immune response to *Campylobacter jejuni* infections in ferrets, we will produce polyclonal anti-ferret IgA. The anti-ferret IgA antibodies will be a useful immunological reagent for the characterization of this *in vivo* model, thus providing insight into the disease process that occurs in man.

Methods & Materials

Immunization of Mice and Rabbits. Female, 18-20 grams, BALB/c mice (Simonsen, Gilroy, CA) were immunized with purified ferret sIgA (prepared as described in the First Year Report) in order to examine their antibody response to the immunogen and to prepare immune sera for the development of the cross-adsorption protocol. The mice were given a primary, intraperitoneal (IP) injection of 25 μ g of sIgA diluted 1/3 in Freund's complete adjuvant. At two week intervals, they were boosted by IP injections of 12.5 μ g of sIgA diluted 1/3 in Freund's incomplete adjuvant. Test bleeds were taken prior to beginning the immunizations and approximately 1 week after each boost. Antibody responses were evaluated by Western blot using a Bio-Rad Mini-PROTEAN II multiscreen apparatus. Ascites production occurred either spontaneously or was induced by intraperitoneal injection of myeloma cells. After two collections of ascites fluid, the mice were sacrificed.

Two New Zealand White Rabbits (8-10lbs; Simonsen, Gilroy, CA) were immunized by subcutaneous and intradermal injections of sIgA in multiple sites on their backs. The primary inoculation contained 250 μ g of sIgA diluted 1/2 in Freund's complete adjuvant. Boosts contained 125 μ g of sIgA diluted 1/2 in Freund's incomplete adjuvant. Test bleeds were taken before the inception of the immunization scheme and after the first boost. Antibody responses were evaluated by Western blot. Once the antibody titers were satisfactory, the blood was collected by cardiac puncture terminal bleeding.

Purification of Ferret IgG. Ferret IgG was purified by affinity chromatography using Staphylococcus Protein A sepharose (Sigma, St. Louis, Mo). Material from the IgG peak fractions

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obtained by size-exclusion chromatography was diluted 9/10 with 1M Tris buffer (TB), pH 8.0. The sample was pumped through a Staph Protein A column (equilibrated against 100mM TB, pH 8.0) at a flow-rate of 0.6ml/minute. Once the sample was loaded, the column was washed with 100mM TB, pH 8.0, until the OD₂₈₀ of the effluent was constant, then subsequently washed with 10mM TB, pH 8.0, until the OD₂₈₀ was 0. The bound IgG was eluted with 100mM glycine-HCl, pH 3.0. Eluted material was immediately neutralized with 1M TB, pH 8.0 (25 μ l of 1M TB per 500 μ l eluate produces a final pH \sim 7.3).

The IgG preparations were further purified by anion exchange chromatography on diethylaminoethyl (DEAE) sepharose to remove contaminant IgA. The DEAE sepharose column was equilibrated against Tris-buffered saline (TBS; 10mM Tris, 100mM NaCl, 0.02% azide), pH 7.5, and the sample was loaded at a flowrate of 0.6ml/min. Under these conditions IgA binds to the DEAE sepharose, but IgG does not. The purity of the IgG preparation was determined by SDS-PAGE and silver stain. Bound IgA was eluted from the column with 10mM TB, pH 8.5, containing 0.3M NaCl.

Cross-adsorption of Mouse Ascites and Rabbit Antisera. Antibodies that cross-reacted with intact ferret IgG were removed by cross-adsorption using ferret IgG immobilized on polyacrylamide beads. The immunoaffinity column was prepared by covalently binding purified ferret IgG to Affi-prep 10 (Bio-Rad, Hercules, CA) as described by the manufacturer. Ascites fluid or rabbit antisera was diluted in 0.1M 3-[N-morpholino]propane-sulfonic acid (MOPS), pH 7.5, containing 0.02% (w/v) sodium azide then passed through the immunoaffinity column at a rate of \sim 0.5ml/min. Alternatively, the ascites fluid or rabbit antisera was batch incubated with the ferret IgG-Affi-prep at 4°C overnight on a rocker. The material that did not bind to the ferret IgG-Affi-prep matrix was tested on Western blots of clarified ferret milk for residual reactivity against intact IgG. The cross-adsorption was repeated until reactivity against sIgA could be detected in the absence of reactivity against intact IgG. Regeneration of the column was accomplished by acid elution (40mM citric acid, 20mM NaCl, pH 3) to remove bound antibody.

The product from the ferret IgG-Affi-prep column was further cross-adsorbed using Western blots of clarified ferret milk. Preparative SDS-PAGE gels containing 10 μ l of clarified ferret milk were prepared (Laemmli, 1970), then the separated milk components were blotted to nitrocellulose (Towbin, 1979). The blots were blocked with 1% skim milk in TBST [10mM Tris, 150mM NaCl, 0.05% (v/v) Tween 20, pH 8.0] for one hour at room temperature, then incubated with the ferret IgG-Affi-prep cross-adsorption product for one hour at room temperature. The efficiency of cross-adsorption was monitored by testing serial dilutions of the cross-adsorbed antisera, or ascites, on blots of clarified ferret milk. The process was repeated until reactivity against sIgA could be detected in the absence of reactivity against non-sIgA components. The final product was tested for specificity by Western blot and enzyme-linked immunosorbent assay

(ELISA; as described in a subsequent section) using purified sIgA and IgG as antigens.

Results

Production of rabbit antisera. At the beginning of the second year of the contract, we completed immunizing two New Zealand White rabbits with purified ferret sIgA. When tested on Western blots of clarified ferret milk, their antisera possessed reactive titers in excess of 1/256,000. The antisera reacted with a variety of milk components including ferret sIgA and IgG as had been reported for the mouse antisera and ascites in the First Year Report. Approximately 130ml of serum was collected from the two rabbits by terminal bleed.

Cross-adsorption of rabbit antisera. We attempted to cross-adsorb the antisera using a ferret IgG-Affi-gel column. This method had successfully cross-adsorbed ascites fluid collected from mice immunized with ferret sIgA (see First Year Report). Unexpectedly, passage of 2mls of rabbit antisera through the column had little or no effect upon reactivity against non-sIgA material. Previously, the column had been used to cross-adsorb mouse ascites after which it was regenerated. It was possible that the regeneration was poor, thus reducing the column binding capacity. This combined with the high titer of the antisera would result in poor cross-adsorption. Repeated cross-adsorption of the rabbit antisera and regeneration of the column produced limited reduction in cross-reactivity, thus indicating that the column was functional. The cross-adsorption product, however, still possessed significant cross-reactivity.

Based upon our results using the regenerated column, we concluded that the preparation of a new column was necessary. To accommodate the high titer of the antisera, we believed that a binding capacity greater than that of our previous column (1mg ferret IgG) would be necessary for effective cross-adsorption. Based upon our yields of IgG from clarified ferret milk, we determined that our remaining stock (96mls) contained approximately 6mg of IgG. Whereas a column containing 6mg of ferret IgG may have been adequate to effectively cross-adsorb the rabbit antisera, production of such a column from our stock of milk would require 100% recovery during purification and 100% coupling efficiency to the Affi-prep matrix. As these efficiencies were not realistic, we needed an alternative source of ferret IgG. Serum IgG concentrations for other mammals are ~10mg/ml (Harlow and Lane, 1988); therefore, during March, we contacted NMRI researchers, who agreed to provide the serum. Due to unexpected complications, delivery of the serum took ~4 months, thus suspending work during this period.

Upon receipt of the ferret serum in July, we purified serum IgG by affinity chromatography on Staph Protein A Sepharose and anion-exchange chromatography on DEAE sepharose. Based upon our yields, the serum contained ~6mg/ml. For preparation of the new column, we used Affi-prep 10 rather than Affi-gel 10, since it can withstand higher pressures, thus

allowing faster flow rates. Six milligrams of ferret IgG was bound to 2ml of Affi-prep. One milliliter of rabbit antisera was cross-adsorbed. Examination of the cross-adsorbed material on Western blots of clarified ferret milk did not reveal any apparent reduction in cross-reactivity against ferret IgG.

Regeneration of the ferret IgG-Affi-prep column released two components as determined by SDS-PAGE: a minor band with the same relative mobility as ferret IgG and a major band with relative mobility of ~ 150 kD. In SDS-PAGE, both mouse and ferret IgG have relative mobilities of ~ 200 kD. We believed that the major component was not rabbit IgG and hypothesized that it was a subunit of the complement cascade component C1q, which is capable of binding to the F_c portion of IgG and has a molecular weight comparable to the unknown component. To determine the identity of the 150kD component, we purified rabbit IgG using Staph Protein A and examined it by SDS-PAGE. The purified rabbit IgG had a relative mobility of ~ 150 kD, thus indicating that the major component eluted from the ferret IgG-Affi-prep column could be rabbit IgG. Western blot analysis using Goat α -rabbit IgG verified the identity of the eluted material as rabbit IgG. Based upon the absorbance at 280nm (Abs_{280}) of the eluted rabbit IgG, at least 2mg had bound to the ferret IgG-Affi-prep column (approximately one-fifth of the estimated amount of IgG in the starting sample). Despite this, no apparent reduction in cross-reactivity was determined, thus indicating that cross-adsorption of the rabbit antisera would be difficult and time-consuming.

Cross-adsorption of mouse ascites. We had previously reported that ascites fluid from mice immunized with purified ferret sIgA was efficiently cross-adsorbed by batch incubation with ferret IgG-Affi-gel 10 matrix (see First Year Report). due to the difficulties in cross-adsorbing the rabbit anti-ferret sIgA antisera, we decided to cross-adsorb some of the mouse ascites and supply this to Naval researchers for use while we continued to cross-adsorb the rabbit antisera.

Repeated cross-adsorption of an aliquot of ascites fluid with ferret IgG-Affi-prep reduced cross-reactivity against intact ferret IgG; however, reactivity against other milk components was not affected. These same components are recognized by a commercially prepared goat α -ferret IgG (not γ -specific; Kirkegaard and Perry Laboratories, Gaithersburg, MD), thus suggesting that they are immunoglobulin degradation products. Upon review of our previous experimental results, it was apparent that reactivity against these components had been reduced but not removed by cross-adsorption. This is in contrast to cross-adsorption of mouse α -ferret sIgA antisera by incubation with unbound ferret IgG which effectively removed reactivity against all ferret milk components other than intact sIgA (see First Year Report). Based upon these results, it is reasonable to conclude that covalent linkage of the IgG to a solid matrix alters the efficiency of cross-adsorption.

Since cross-adsorption with ferret IgG bound to Affi-prep did not remove reactivity against possible immunoglobulin degradation products, we further cross-adsorbed the ascites using Western blots of clarified ferret milk. Prior to adsorption, the portion of the blot containing sIgA was removed to avoid reduction of sIgA-specific reactivity. Cross-adsorption by this method significantly reduced reactivity against immunoglobulin degradation products without significantly reducing reactivity against sIgA. Repeated cross-adsorption produced a sIgA-specific product. Following the fifth cross-adsorption, the ascites possessed very little reactivity against non-sIgA milk components when tested on Western blots at dilutions of 1/500 and 1/1,000 (figure 1).

To confirm sIgA specificity of the cross-adsorbed ascites, we tested for reactivity against purified ferret IgG and sIgA on Western blots and in ELISA. On Western blots, a 1/1,000 dilution of the cross-adsorbed ascites did not react with purified IgG, but was strongly reactive against sIgA (figure 2). In ELISA, a 1/500 dilution did not possess detectable reactivity against IgG, while it was strongly reactive against sIgA. We sent 0.5ml of the cross-adsorbed material to the Navy in December.

2. Extraction and Purification of Lipopolysaccharide

Lipopolysaccharide (LPS) is present in the cell walls of all gram-negative bacteria. LPS is a heat-stable toxin as well as a major bacterial antigen. It has toxic, pyrogenic, and mitogenic properties. LPS induces the release of tumor necrosis factor by macrophages and acts as an immunoadjuvant. It is a virulence factor for pathogenic gram-negative bacteria and is responsible for the induction of fever and endotoxic shock during infection.

LPS is composed of three basic subunits: lipid A, the core, and the O-specific side chain. Lipid A is a phosphorylated glucosamine disaccharide esterified with fatty acids. The toxic properties of LPS are due to this portion of the molecule. The core is an oligosaccharide which commonly contains heptose and 3-deoxy-D-manooctulosonic acid (KDO). This portion, along with the O-specific side chain, define the antigenic portion of LPS. The O-specific side chain is a polysaccharide composed of repeating tri-, tetra-, or pentasaccharide subunits. The composition of the O-side chain is responsible for the antigenic specificity of LPS. Serotyping based upon this structure has been used extensively for the identification of different bacterial strains.

Despite being a major cell-surface antigen, the role of α -LPS antibodies in the development of immunity against infection by enterics such as *Shigella* is unknown. Some studies exploring this relationship in shigellosis have shown a direct relationship between the levels of α -LPS antibodies and protection from infection (Cohen et al., 1988, Cohen et al., 1991); whereas, other studies, such as that of Oberhelman et al. (1991), have suggested that antibodies against invasion

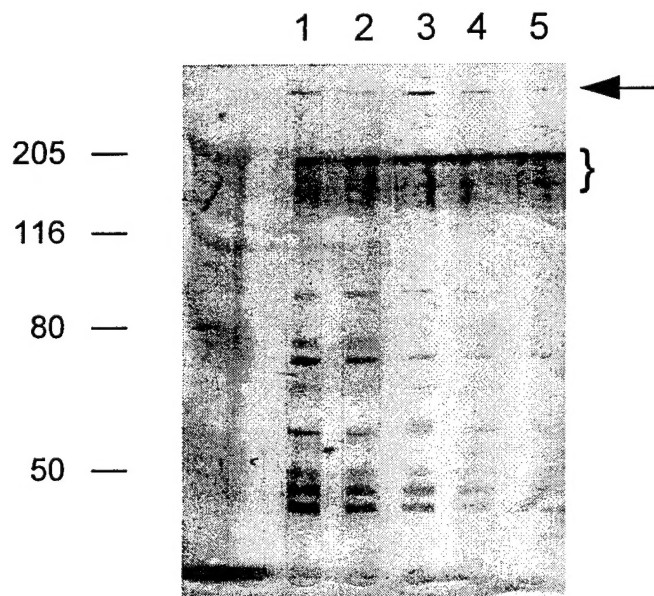


Figure 1. Cross-adsorption of mouse ascites containing α -ferret sIgA antibodies. Blot contains clarified ferret milk. Ferret sIgA denoted by arrow. Test sera are as follows: Lane 1) Starting ascites (Ferret IgG -Affi-prep adsorbed) at 1/1,000 dilution; Lane 2) Starting ascites at 1/2,000 dilution; Lane 3) Final product (cross-adsorbed 5x with nitrocellulose blots) at 1/250 dilution; Lane 4) Final product at 1/500 dilution; Lane 5) Final product at 1/1,000 dilution. Reactivity marked by bracket due to staining by secondary antibody (goat α -mouse IgG). Sizes of molecular weight standards in kilodaltons as indicated.

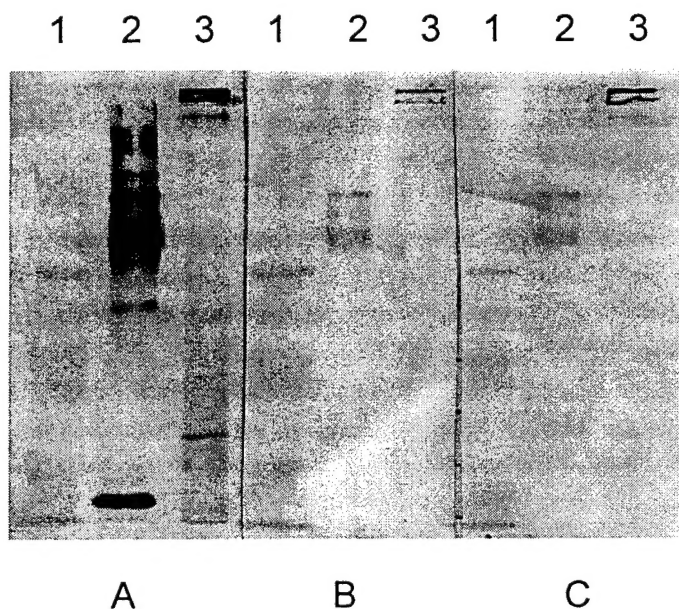


Figure 2. Specificity of the cross-adsorbed mouse ascites. For each blot, lane 1 contains pre-stained molecular weight standards, lane 2 contains purified ferret IgG, and lane 3 contains purified ferret sIgA. Blot A was developed using unadsorbed ascites fluid. Blot B was developed with secondary antibody (goat α -mouse IgG) only. Blot C was developed with the cross-adsorbed ascites.

plasmid antigens, rather than those against LPS, are required for protection. Therefore, the determination of antibody titers against LPS should be included as part of epidemiological analyses of infection rates or evaluations of enteric vaccines. We will extract LPS from *Shigella sonnei* 53LB, *S. flexneri* 2457, and *Campylobacter jejuni* 81176 for the Navy's use in such studies.

Methods & Materials

Organisms and Culture Conditions. *Campylobacter jejuni* 81176 was provided by researchers at the Naval Medical Research Institute in Bethesda, Maryland. For cultivation, frozen stocks were streaked onto Mueller-Hinton (M-H) plates and incubated under microaerophilic conditions in Campy Pouches (BBL, Cockeysville, MD) at 37°C overnight. The next day, the plates were flooded with fresh BHI broth containing 1% yeast extract (BHI-YE), and the cells were suspended using a sterile loop. Biphasic media was inoculated with the cell suspension as described by Lee et al. (1993). The optical density at 600nm (OD₆₀₀) of the cell suspension was adjusted to 0.05 by dilution in fresh BHI-YE. Mueller-Hinton agar (2ml) in 25cm² tissue culture flasks were overlaid with 8ml of the cell suspension, and the flasks were incubated aerobically at 37°C overnight. Five hundred milliliters biphasic cultures (100ml M-H agar overlaid with 400ml of BHI-YE) were inoculated with cells from the 10ml biphasic culture to produce a starting OD₆₀₀ of 0.02. The biphasic cultures were incubated overnight at 37°C with shaking (100 rpm). Cells were collected by centrifugation (2,900xg, 30min), washed twice with sterile nanopure water, killed by addition of formaldehyde (0.5% final concentration), washed once with sterile nanopure water, then stored at -20°C until extracted.

Alternatively, cell suspensions prepared from the initial M-H plates were spread onto additional M-H plates. Plates were inoculated with 0.1-0.2ml of the cell suspension (OD₆₀₀ ~ 1) then incubated at 37°C under microaerophilic conditions in a GasPak container (Becton Dickinson, Cockeysville, MD) for 48 hours. After incubation, the plates were flooded with sterile nanopure water, and the cells were scraped from the agar using a loop. Cells were pelleted by centrifugation then killed and stored as described above.

Extraction of LPS by modified phenol-water method. We experimented with two methods for extracting *C. jejuni* LPS. In the first method (Chester and Murray, 1975), cells were treated with pronase prior to LPS extraction by the phenol-water method of Westphal and Jann (1965). Killed cells were suspended in sterile nanopure water, and pronase was added to a final concentration of 0.2mg/ml. The mixture was incubated at 37°C for six hours on a rotary shaker. The pronase-treated cells were pelleted by centrifugation (2,900xg) for thirty minutes at 15°C, washed once with sterile nanopure water, then stored at -20°C until extracted.

To extract the LPS, we suspended pronase-treated cells were suspended in sterile deionized

water that had been heated to 68°C (5mls water per gram of cells). The cell suspension was added to an equal volume of 99% phenol. The phenol was melted by heating to 68°C, and then the mixture was incubated for 15 minutes with agitation (110 rpm) at 68°C. After incubation, the mixture was cooled in an ice water bath to facilitate phase separation and then centrifuged (2,900xg) for one hour at 10°C. Centrifugation produced three distinct phases: an upper aqueous layer, a middle layer of insoluble material, and a bottom layer of phenol. The aqueous layer was collected, and the phenol layer was re-extracted by addition of a second volume of sterile, deionized water. The aqueous phases from the two extractions were pooled, then dialyzed (dialysis tubing MWCO 6,000-8,000) against deionized water for 2 days at 4°C to remove residual phenol. After dialysis, the dialysate was centrifuged (8,150xg) at 4°C to remove particulate material. The supernatant was collected, then centrifuged at 80,000xg for 2 hours at 15°C. The supernatant was discarded, and the pellet of LPS was resuspended in fresh deionized water, lyophilized, then stored at -20°C until analyzed.

Detergent extraction of LPS. The second LPS extraction method utilized the detergent sodium dodecyl sulfate (SDS) to solubilize LPS (Darveau and Hancock, 1983). Formalin-killed cells were suspended in 10mM TB, pH 8.0, containing 2mM MgCl₂ (5g wet weight cells per 8-10ml buffer). The cell suspension was placed in an ice-bath then sonicated with twelve 30 second bursts using a microprobe at a setting of 4.5. After sonication, the suspension volume was adjusted to 15ml with fresh 10mM TB containing 2mM MgCl₂. DNase and RNase were added to final concentrations of 200 and 50µg/ml respectively, and the cells were incubated for 2 hours at 37°C. After the incubation was completed, 5ml of 0.5M tetrasodium EDTA (Na₄EDTA) in 10mM TB, 2.5ml of 20% (w/v) SDS in 10mM TB, and 2.5ml of 10mM TB were added. The mixture was vortexed for 3 minutes then centrifuged at 50,000xg for 30 minutes at 20°C. The supernatant was collected, and pronase was added to a final concentration of 200µg/ml. The sample was then incubated at 37°C with constant shaking overnight.

The following day, any precipitate that had formed was removed by centrifugation at 1,000xg for 10 minutes. The supernatant was mixed with 2 volumes of 0.376M MgCl₂ in absolute ethanol, and the mixture was cooled to 0°C in a dry ice bath. The precipitate was collected by centrifugation (12,000xg) for 15 minutes at 0-4°C. The pelleted LPS was suspended in 25ml of 10mM TB containing 2% (w/v) SDS and 0.1M Na₄EDTA. If necessary, the sample was sonicated to form a uniform suspension. The solution was incubated at 85°C for 10-30 minutes then cooled to room temperature, and if necessary, the pH was adjusted to 9.5. Pronase was added to a final concentration of 25µg/ml, and the sample was incubated overnight at 37°C with constant agitation.

On the third day of the extraction, the LPS was precipitated with ethanol (described above), and the pellet was suspended in 15ml of 10mM TB and sonicated. If insoluble Mg²⁺-

EDTA crystals were present, they were removed by centrifugation (1,000xg). $MgCl_2$ was added to a final concentration of 25mM, then the sample was centrifuged at 200,000xg for 2 hours at 15°C. The resultant LPS pellets were suspended in nanopure water then lyophilized.

Analysis of LPS. LPS preparations were analyzed for purity by SDS-PAGE in 14% slab gels containing 4M urea. LPS was visualized by silver stain as described by Tsai and Frasch (1982). Gels were fixed overnight in 40% ethanol-5% acetic acid. The next day, gels were incubated in 40% ethanol-5% acetic acid containing 0.7% periodic acid for five minutes to oxidize the LPS then washed three times (15 minutes/wash) with nanopure water. The gels were then stained using a commercially-available silver stain kit (Sigma, St. Louis, MO) as described by the manufacturer. Contaminant proteins were visualized in gels by Coomassie stain. Total protein in the preparation was determined using the Bio-Rad (Hercules, CA) detergent-compatible protein assay. Contaminant nucleic acid was detected by UV absorbance.

Isolation and characterization of outer membrane vesicles. We prepared and examined *C. jejuni* outer membrane vesicles as a possible alternative source of LPS. Crude outer membrane vesicle preparations were produced using a modification of the method of Gu and Tsai (1991). *C. jejuni* 81176 cells were grown on M-H agar under microaerophilic conditions at 37°C for 72 hours then suspended in BHI-YE. A 100ml biphasic culture (90ml BHI-YE over 10ml M-H agar layer) was inoculated with the cell suspension to form a final OD_{600} of 0.1 then incubated at 37°C for 48 hours with constant shaking (100rpm). The cells were pelleted by centrifugation (3,128xg) at 15°C for 30 minutes, and the supernatant was collected then passed through a 0.45 μ syringe filter. The filtrate was buffer exchanged (against sterile nanopure water) and concentrated in a Centriprep-100 (Amicon, Beverly, MA). The concentrate was analyzed by SDS-PAGE using silver and Coomassie stains.

Results

In October, 1993, work on this task was suspended indefinitely due to funding reductions. At the end of April, 1994, we were informed by Naval administrators that some funding had been reinstated (all funding was restored in July, 1994), and work on this task was resumed at the beginning of May.

Production of cell mass for extraction. Initially, we attempted to cultivate *C. jejuni* 81176 in biphasic media. Unfortunately, we obtained low yields of cell mass using this system. From 1.6 liters of broth overlay from 48 hours biphasic cultures, we collected ~0.4g of ethanol-dried *C. jejuni* 81176 cell mass. In comparison, we collected ~3g of ethanol-dried *Shigella sonnei* 53LB per liter of broth culture following 24 hours incubation. Assuming a 3% LPS yield (based upon *Shigella* yields) and a 70% cell water content (Ingraham et al., 1983), production of the requisite

200mg LPS would have required ~ 22g of ethanol-dried cells. Production of this amount of cell mass would have required ~ 110 days of continuous cultivation, thus making biphasic cultivation impractical.

As a possible alternative source of LPS, we had been propagating an aerotolerant strain of *C. jejuni* 81176 that was isolated during a purity check for aerobic contaminants in a biphasic culture. The aerotolerant organism was easier to cultivate (i.e. - did not require biphasic media) and produced three-fold greater cell mass than the oxygen sensitive parent strain. For these reasons, we extracted and analyzed LPS from the aerotolerant strain and compared it with that of the parent to ascertain whether the variant was a suitable substitute. SDS-PAGE analysis revealed differences in composition between the LPS of the aerotolerant and parental strains, thus indicating that the aerotolerant isolate could not be substituted for the microaerophilic parent strain.

Subsequently, we explored an alternative cultivation method. We compared cell mass yields from cultivation of *C. jejuni* 81176 on M-H agar (48 hours, 37°C, microaerophilic conditions) with that of biphasic media (48 hours, 37°C, aerobic conditions). We determined that the equivalent cell mass of a 500ml biphasic culture (400ml broth overlay, 100ml agar base) could be collected from 4 agar plates. This made cultivation of the necessary amounts of cell mass feasible. Due to the limited capacity (36 plates) of the GasPak container (BBL, Cockeysville, MD) that was necessary for maintenance of microaerophilic conditions, however, the collection was still time-consuming.

Extraction of *Campylobacter jejuni* LPS. Prior to the suspension of work, we performed a preliminary extraction using the phenol-water method of Westphal and Jann (1965). The extraction was poor with a yield of less than 0.05% (wt LPS/dry cell wt). This was not surprising since *C. jejuni* strains contain rough-type LPS which is not efficiently extracted by the phenol-water method.

Upon provision of additional funds, we experimented with the method of Næss and Hofstad (1984) which has been used to successfully extract LPS from strains of both *C. jejuni* and *C. coli*. Cells were treated with pronase prior to LPS extraction in order to remove a glycoprotein microcapsule which inhibits efficient extraction (Conrad and Galanos, 1990). Initial extractions yielded products that were too small to accurately quantitate; however, the yields appeared to be unchanged, thus indicating that an alternative method was necessary.

Originally, we planned to use the method of Galanos et al. (1969) in which rough LPS is extracted with a phenol-chloroform-petroleum ether mixture. The efficiency of extraction, however, varies from strain to strain and, in some cases, is less than that of the Westphal phenol-

water method (Perez and Blaser, 1985). For this reason, we chose to experiment with the method of Darveau and Hancock (1983) which produced 3-4% yields from strains of *C. fetus* that were not extractable with either the Galanos or Westphal methods (Perez and Blaser, 1985). The method utilizes the detergent sodium dodecyl sulfate (SDS) to solubilize LPS. Contaminant proteins and nucleic acids are enzymatically digested, and the LPS is purified by ethanol precipitation.

Using the SDS-extraction method, our initial yields were approximately 0.6% which was too low for feasible production of 200mg LPS; however, further optimization of the extraction protocol increased the yield to a maximum of 5% with an average of 3%. Characterization of the extraction product has shown that the LPS is of low molecular weight (figure 3) which is consistent with rough type LPS. Additionally, the product does not contain detectable protein by Coomassie staining of SDS-PAGE gels, and contaminant nucleic acid cannot be detected by UV absorbance. By the end of the second year, we had collected 173mg of LPS and had begun cultivation of cells for production of the remaining 23mg.

Analysis of outer membrane vesicles. While optimizing the SDS-extraction protocol, we explored the possibility that *C. jejuni* 81176 secretes LPS in biphasic cultures. This study was prompted by an article by Gu and Tsai (1991) in which they reported purifying LPS from outer membrane vesicles (OMV's) secreted by *Neisseria meningitidis* in broth culture. Comparison of LPS from *N. meningitidis* OMV's and cells from 1ml of broth culture revealed approximately 2.5 times as much LPS in OMV's as in cells (47.9 μ g vs 18.5 μ g). The LPS from the two sources were indistinguishable by SDS-PAGE and produced similar results when tested in the *Limulus* amoebocyte lysate assay. If *C. jejuni* 81176 secreted similar amounts of LPS, it would be possible to collect the required 200mg from culture supernatants.

Approximately 120ml of broth from a 48 hour biphasic culture was concentrated to 2ml by ultrafiltration. The concentrated broth was analyzed by SDS-PAGE and silver stain. Our results suggested that LPS was present in the broth. Based upon visual comparison with purified *C. jejuni* LPS standards, the concentrated material contained approximately 1 μ g per 8 μ l. Since the broth had been concentrated ~60-fold, the starting material contained ~2 μ g per ml, which would necessitate the collection of 100 liters of broth for the preparation of 200mg LPS (assuming 100% recovery). For this reason, further purification and analysis of the secreted material was not performed.

3. Enteroaggregative *Escherichia coli* Heat-Stable Toxin 1 (EAST1)

Due to the reduction in funding for this project and the lack of satisfactory recombinant organisms for production of EAST1, this portion of the project was suspended in October, 1993. Although funds were restored, suitable recombinants have not been developed by Naval

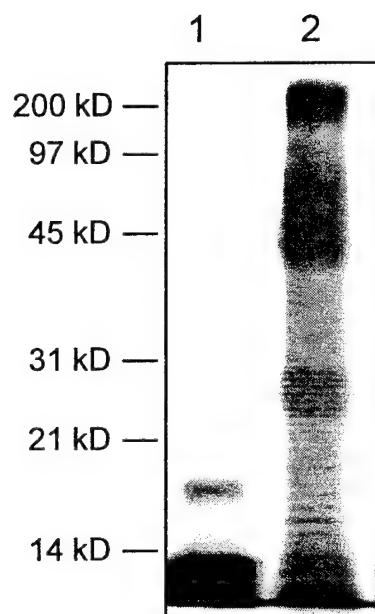


Figure 3. SDS-PAGE of purified *Campylobacter jejuni* 81176 LPS prepared by the SDS extraction method. Lane 1) 5 μ g of purified *C. jejuni* LPS. Lane 2) 5 μ g of smooth LPS from *Shigella sonnei* 53LB. Approximate positions of molecular weight markers are indicated.

researchers; therefore, in October, 1994, Captain A.L. Bourgeois indicated that reinstatement of this task is improbable.

4. Type-Specific *Campylobacter* Flagellin Epitopes

Campylobacter coli and *Campylobacter jejuni* are significant enteric pathogens of humans (Blaser and Reller, 1981; Griffiths and Parks, 1990; Walker et al., 1986). Infections involving these motile, gram-negative bacteria most commonly manifest as gastrointestinal disorders characterized by fever, nausea, abdominal cramps, and diarrhea, although uncommon, extraintestinal disorders such as meningitis, cholecystitis, and urinary tract infections have been reported. In 1989, thirty thousand cases of enteritis were attributed to campylobacter infection in England and Wales. The prevalence of campylobacter infections usually exceeds that of the better known enteric pathogens of the genus *Salmonella* (Griffiths and Parks, 1990).

The existence of various campylobacter virulence factors such as adhesins, enterotoxins, and cytotoxins have been investigated, yet the pathogenic mechanism remains poorly defined. The campylobacter flagellin has been the subject of great interest as a pathogenic determinant. These structures are the immunodominant protein antigens in human infection (Logan et al., 1987) and have been shown to be important for colonization of the mucosal epithelia by the pathogen. Additionally, antigenic variation of the flagellar epitopes has been reported (Harris et al., 1987); this may be significant to the organism's ability to avoid host defenses. Harris et al. (1987) isolated two such antigenic variants of *C. coli* VC167 (VC167T1 and VC167T2). Recently, it was reported that the antigenic variations seen between these two variants are due to undefined post-translational modifications (Alm et al., 1992). The purpose of this portion of the project is to develop monoclonal antibodies which recognize these post-translational modifications to aid in the determination of their structure.

Methods & Materials

Microorganisms and purified flagellin. *Campylobacter coli* strains VC167T1, VC167T2, and VC167T2316-7A were provided by Dr. Trevor Trust of the University of Victoria (British Columbia, Canada). *C. jejuni* 81176 was provided by researchers at NMRI (Bethesda, MD). Purified *C. coli* VC167T2 flagellin was prepared by Dr. Mary Power, a post-doctoral fellow in Dr. Trust's laboratory. Bacteria were grown on M-H agar in Campy Pouches (BBL, Cockeysville, MD) at 37°C. For the preparation of killed-cell suspensions, bacteria were scraped from plates and suspended in sterile PBS (4.3mM Na₂HPO₄, 2.7mM KCl, 137mM NaCl, pH 7.2). Formalin (37% formaldehyde) was added to a final concentration of 0.5% formaldehyde, and the suspension was left at room temperature for 1-2 hours. Killed cells were pelleted by centrifugation (2,900xg), washed once with PBS-azide, then resuspended in a small volume of PBS-azide. The OD₆₀₀ of the

final cell suspension was determined using a Spectronic 20 spectrophotometer. Cell suspensions were stored at 4°C.

Immunization of mice. Mice were bled prior to injection, and their sera were tested by ELISA for reactivity against whole cells and purified flagellin. Female BALB/c mice (18-20 grams; National Cancer Institute) were given a primary, intraperitoneal injection of 50µg of purified T2 flagellin mixed with Freund's complete adjuvant (1 volume flagellin per two volumes adjuvant). Mice were boosted 3 times at two weeks intervals by intraperitoneal injection of 25µg of flagellin (mixed 1:2 in Freund's incomplete adjuvant). The mice were bled one week after each boost. Anti-campylobacter serum antibody titers were determined using a whole cell (ELISA). The mice were given a final intravenous boost of 25µg of flagellin three days prior to being sacrificed for production of hybridomas.

Production of α -flagellin MAbs. Mice were sacrificed by cervical dislocation, and their spleens were removed aseptically. The spleens were placed in a sterile petri dish with 7mls of RPMI media (Bio-Whittaker, Walkersville, MD). Scar tissue was removed, then the spleen cells were teased apart. Clumps of cells were allowed to settle out of the cell suspensions by gravity. The splenocytes were fused with P3X63-Ag8.653 myeloma cells using 50% (v/v) polyethylene glycol. Resultant hybridomas were selected using HAT medium. When adequate cell growth was achieved, culture supernatants were screened for IgG production and strain specificity by ELISA.

Enzyme-linked immunosorbent assay. A whole cell ELISA was developed based upon the method of Van den Bosch et al. (1993). Suspensions of formalin killed cells were diluted in fresh PBS-azide to an OD₆₀₀ of 0.02. Fifty microliters of this cell suspension was added to each well of a 96 well Immulon II microtiter plate (Dynatech, Chantilly, VA). The cells were dried onto the plates by incubation at 37°C overnight. The following day, the wells were washed twice with distilled water. Each well was blocked by addition of 200µl of 3% bovine serum albumin (BSA) in TBS-azide (10mM Tris-HCl, 150mM NaCl, 0.02% sodium azide), pH 7.0, and incubated at room temperature for 1 hour. After the wells were blocked, they were washed twice with TBS-azide, then 50µl of the test antibodies (diluted in 3% BSA in TBS-azide if necessary) was added to the appropriate well. Plates were covered with parafilm and incubated at 37°C for 1 hour. After incubation, the wells were washed 4 times with TBS-azide. Fifty microliters of an appropriate alkaline phosphatase-labeled secondary antibody (diluted in 3% BSA in TBS-azide) was added, and the plate was incubated at 37°C for 1 hour. Once the incubation was completed, the wells were washed four times with TBS-azide, then 200µls of a 1mg/ml p-nitrophenyl phosphate solution in alkaline phosphatase development buffer (100mM Tris, 100mM NaCl, 5mM MgCl₂·6H₂O, 0.02% NaN₃, pH 9.5) was added. The plates were developed at room temperature. Color development was measured by absorbance at 405nm using a Dynatech MR5000 plate reader.

For the determination of IgG production, the same protocol was followed except that unlabelled goat α -mouse IgG was dried onto the walls of the wells rather than whole cells. For determination of α -flagellin reactivity, wells were coated with 0.25 μ g of purified T2 flagellin.

Immunofluorescence microscopy. Ten microliters of formalin-killed cell suspensions ($OD_{600}=0.02$) were dried onto fluorescent microscope slides at 37°C. The dried cells were overlaid with 10 μ l of the test antibody, then incubated at 37°C in a humidified chamber for 30 minutes. After the incubation was complete, the slides were washed with TBS-azide, pH 7.0, containing 1% Tween-20, then rinsed with TBS-azide without detergent. Excess liquid was removed, and the cells were overlaid with 10 μ l of an appropriate FITC-conjugated secondary antibody. Plates were incubated for 30 minutes at 37°C with humidity. After labeling was completed, the cells were washed with TBS-azide, then rinsed with deionized water. The slides were dried, and coverslips were mounted with glycerol. Cells were examined for immunofluorescence using a Zeiss Axioskop 20 equipped for epi-fluorescence.

Flow cytometry analysis. The strain specificities of selected MAbs from the first and second fusions were further tested by flow cytometry. Formalin-killed *C. coli* VC167T1 or VC167T2 cells ($\sim 10^9$ cells based upon microscopic counts) were incubated with MAbs in tissue culture supernatants (200 μ l) for 1 hour at 37°C or overnight at 4°C (total reaction volume of 400 μ l). After incubation, cells were pelleted by centrifugation (12,000xg) and washed twice with TBS-azide. The washed cells were suspended in 200 μ l TBS-azide, mixed with 100 μ l of fluorescein-labelled goat α -mouse IgG (Caltag, South San Francisco, CA), then incubated for 1 hour at 37°C. After incubation, the cells were washed twice with TBS-azide then suspended in 500 μ l of PBS. Samples were analyzed flow cytometry by the Research Corporation of the University of Hawaii.

Results

Screening of hybridomas. During the second year of the contract, we completed three myeloma-splenocyte fusions and screened the resultant hybridomas. No T2-specific hybridomas were isolated. In the first fusion, few MAbs were identified; this was unexpected as the mouse that was used for the fusion possessed a reactive serum titer of $>1:160,000$ in the whole cell ELISA prior to being sacrificed. In the second and third fusions, we screened approximately 300 hybridomas per fusion for production of T2-specific MAbs. The number of hybridomas that can be screened per fusion is limited by the requirement of individual hybridomas in wells for detection of strain-specific MAbs and avoidance of false-negatives. From the three fusions, 13 hybridomas have been saved. They were originally chosen based upon either their greater reactivity against T2 cells relative to T1 cells or their strong signal production and possible utility as genus- or species-specific probes. Initially, some of the MAbs produced readings that were comparable to cross-adsorbed polyclonal rabbit antisera that are specific for either T1 or T2;

however, the T2-specificity diminished during expansion of the hybridoma cultures. Subsequent sub-cloning did not produce clones with the initial T2-specificity. The reactivities of the stored hybridomas in the whole cell ELISA are presented in table 1.

The reactivities of 6 of the MAbs on Western blots of whole cell lysates are presented in figure 4. Three MAbs produced by hybridomas CCA 079, CCB 227, and CCC 212 are reactive against flagellin, although they also label other bands. We have prepared ascites using these three hybridomas and intend to explore their potential utility for flagellin purification by immunoaffinity chromatography. Five of the remaining 7 MAbs have also been tested on Western blots; however, this experiment must be repeated.

During July, Dr. Ogata was to attend the Naval conference on *Campylobacter* in Cairo, Egypt, during which discussions with Naval researchers regarding possible changes in protocol were to be held. Due to illness, Dr. Ogata was unable to attend. Captain Bourgeois of NMRI felt that in person discussions would be beneficial to the successful completion of this portion of the project; therefore, he was to schedule a visit by Dr. Ogata to the Navy research facilities in Maryland during September. Due to serious medical emergencies in both Captain Bourgeois' and Dr. Ogata's families, travel could not be arranged, and discussions on possible changes in protocol were not resumed until November. Further immunizations had been suspended in July due to the possible changes in protocol; therefore, following discussions in November, immunization was resumed. By the end of the second year, we had begun immunizing two additional mice which will be used during the current no-cost extension of the contract.

MAb CCB 227. Of the 17 hybridomas that have been saved, MAbs produced by CCB 227 have been best characterized. Originally, this MAb was thought to be T2-specific as its reactivity in the whole cell ELISA against T2 was as much as 6 times greater than against T1. Analysis by flow cytometry (figure 5) supported these findings; however, testing in a sandwich ELISA for reactivity against flagellin and on Western blots of whole cell lysates demonstrated that the MAb reacted with flagellin from both T1 and T2 cells. These findings indicated that the MAb does not recognize the T2-specific post-translational modification of flagellin.

In immunofluorescence microscopy, MAb CCB 227 labelled the entire surface of T2 cells, but not that of T1 cells. Therefore, we attempted to isolate the cell surface epitope by immunoprecipitation; however, this approach was unsuccessful. On Western blots of whole cell lysates, MAb CCB 227 labelled several bands with molecular weights less than 50kD in T2 cells as well as cells of T2316-7A, a T2 mutant which lacks the T2-specific flagellar epitope (figure 6). The MAb did not recognize similar bands in T1 cells. When tested on blots of glycine-HCl extracts of T2 and T1 cells, only flagellin was labelled (figure 6), thus indicating that the T2-unique components are not flagella-related. Konkel et al. (1990) reported the development of a

Table 1. Reactivities of MAbs produced by hybridomas saved from first three fusions. Relative reactivities on the different cell types cannot be compared between hybridomas (e.g. - reactivity of CCB 227 on T2 is not twice that of CCA 079 on T2). Cross-adsorbed polyclonal antisera (LAH-1 and LAH-2) are included as references for strain-specific readings. LAH-1 is T1-specific, while LAH-2 is T2-specific.

| MAb | Isotype | Whole Cell ELISA <i>C. coli</i> VC167 | | | <i>C. jejuni</i> 81-176 |
|---|------------------|--|--------|-----------|----------------------------|
| | | T1 | T2 | T2316-7A* | |
| CCA 079 | IgG ₁ | + | + | + | - |
| CCA 086** | nd | - | - | nd | nd |
| CCA 099** | nd | - | - | nd | nd |
| CCA 101 | IgM | weak + | weak + | nd | nd |
| CCA 108** | nd | - | - | nd | nd |
| CCB 227 | IgG ₃ | + | 2+ | 2+ | - |
| CCC 004 | IgM | + | + | + | 2+ |
| CCC 051 | IgG ₃ | + | 2+ | 2+ | - |
| CCC 059 | IgM | + | + | + | + |
| CCC 171 | IgM | + | 2+ | 2+ | 2+ |
| CCC 212 | IgG ₃ | + | + | + | + |
| CCC 214 | nd | + | + | + | 2+ |
| CCC 224 | IgM | + | + | + | 2+ |
| LAH-1 | Polyclonal | 4+ | + | + | + |
| LAH-2 | Polyclonal | + | 5+ | + | + |
| * = T2 mutant lacking post-translational modification ** = reactive when incubated with cells in suspension; none T2-specific nd = not determined | | | | | |

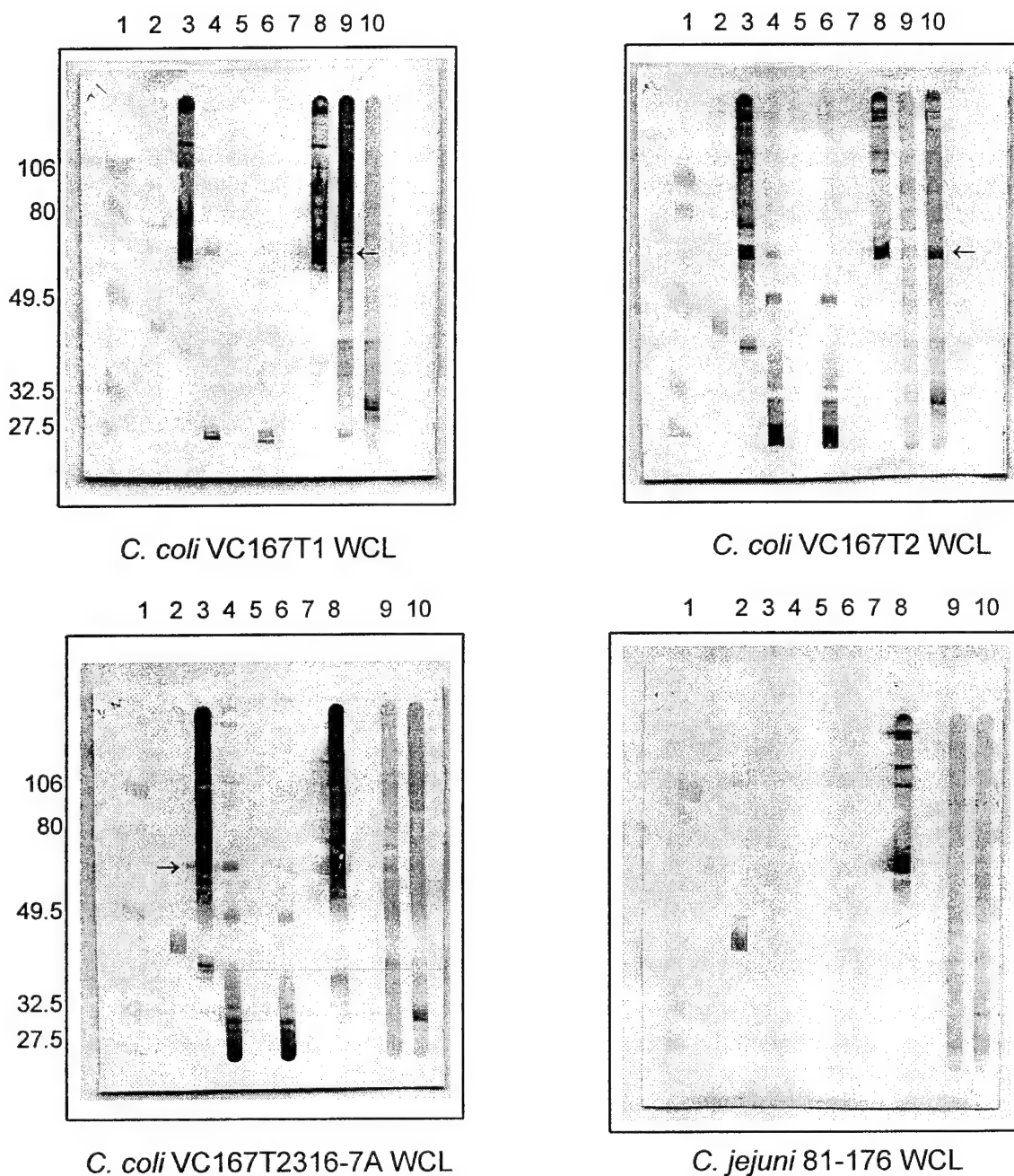


Figure 4. Reactivities of selected MAbs on Western blots of whole cell lysates (WCL). For each blot, the bacterial strain that was used is indicated. MAbs used in each lane are as follows: 1) MW Stnds. (no MAb); 2) CCA 086; 3) CCA 079; 4) CCB 227; 5) CCC 004; 6) CCC 051; 7) CCC 173; 8) CCC 212; 9) T1 specific polyclonal rabbit antisera (LAH-1); 10) T2-specific polyclonal rabbit antisera (LAH-2). The locations of the flagellin bands for the *C. coli* strains are indicated by the arrows. MAb CCC 173 stopped producing reactive antibodies and is not included in table 1.

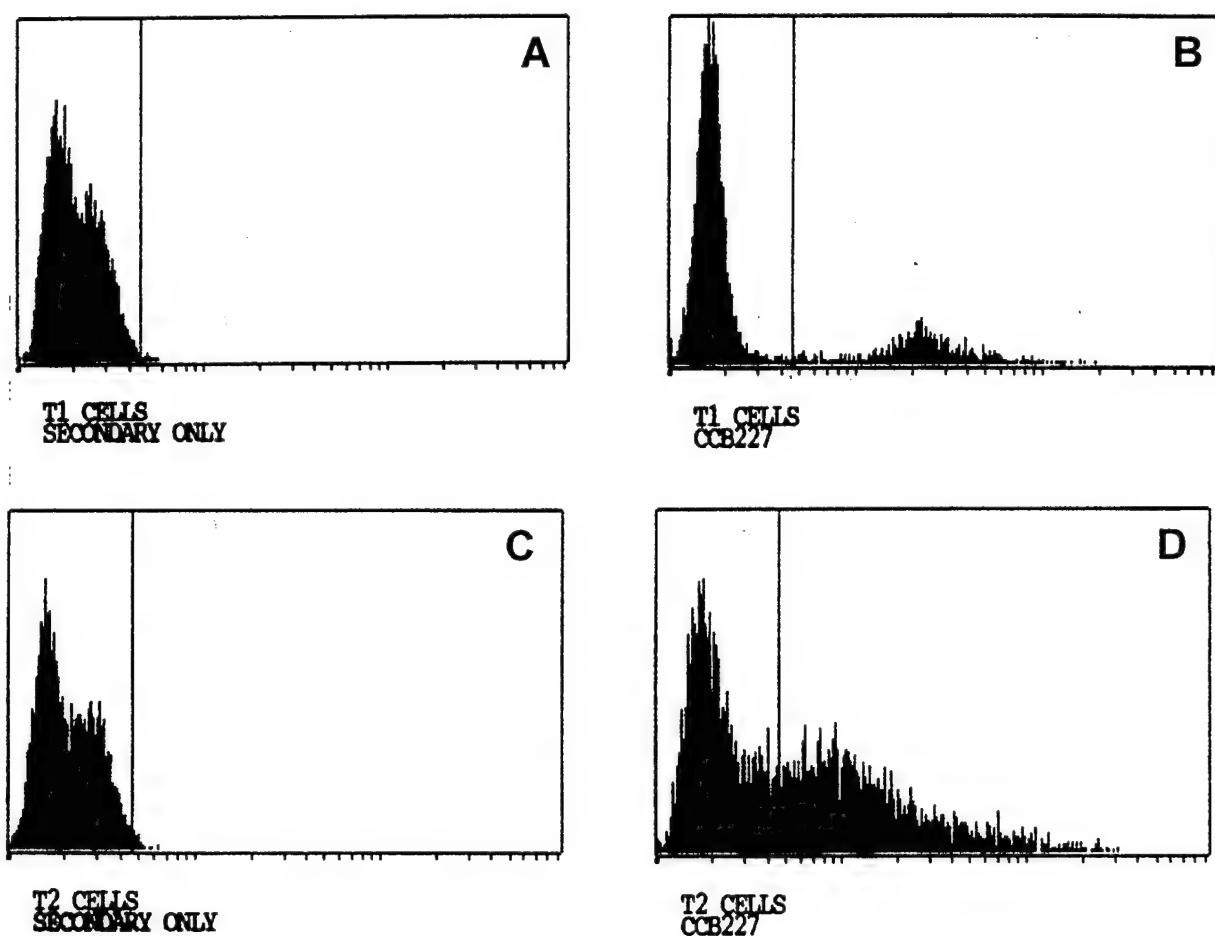


Figure 5. Results from flow cytometry analysis of MAb CCB 227. The MAb labels much of the population in a T2 cell preparation, but only a small portion of T1 cells are labelled. A) T1 cells stained with secondary antibody (FITC labelled goat α -mouse IgG) only; B) T1 cells stained with MAb CCB 227; C) T2 cells stained with secondary only; D) T2 cells stained with MAb CCB 227. For each plot the Y-axis is the number of cells counted, and the X-axis is the amount of fluorescence (increases from left to right).

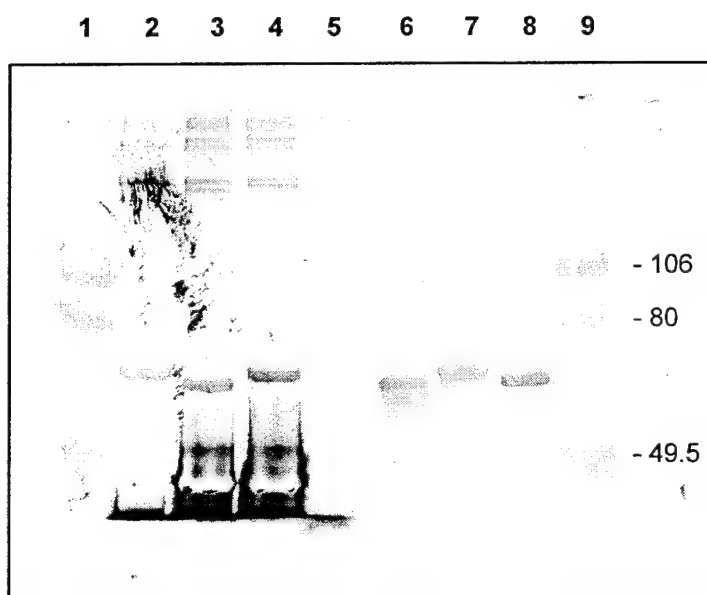


Figure 6. Reactivity of MAb CCB 227 on Western blots of whole cell lysates and glycine-HCl extracts of *C. coli* and *C. jejuni*. Lanes 1 & 9) pre-stained MW stdns (106K, 80K, 49.5K); Lane 2) *C. coli* VC167T1 whole cell lysate (WCL); Lane 3) *C. coli* VC167T2 WCL; Lane 4) *C. coli* VC167T2316-7A WCL; Lane 5) *C. jejuni* 81176 WCL; Lane 6) Purified T2 flagellin; Lane 7) Glycine-HCl extract from T1; Lane 8) Glycine-HCl extract from T2.

MAB against *C. jejuni* which also recognized an epitope present on both the cell surface and flagella. They determined that the MAB recognized an invasin and demonstrated that it inhibited invasion by *C. jejuni*. Therefore, we tested CCB 227 for the ability to block invasion of HeLa cells by *C. coli* VC167T2. Unfortunately, the bacteria bound poorly to the cells, and further testing was not performed due to the reinstatement of suspended portions of the project.

III. Plans for Present Quarter

1. Production of Anti-Ferret IgA Antibodies

Although we successfully cross-adsorbed the mouse ascites using a ferret IgG immunoaffinity column and Western blots of ferret milk, the method is not feasible for cross-adsorbing the rabbit antisera. To cross-adsorb 0.18ml of undiluted ascites, a 6cm x 9cm nitrocellulose blot was used. The rabbit antisera has a reactive titer in excess of 1/256,000 when tested on Western blots of ferret milk; this is greater than 30-fold higher than the titer of the ascites. Therefore, cross-adsorption of an equivalent volume of rabbit antisera would require 30 times as much nitrocellulose. Since the contract requires conjugation of the α -sIgA to alkaline phosphatase, cross-adsorption of at least 2 ml of antisera (~20mg IgG) will probably be necessary. This will require ~300 times the amount of nitrocellulose required for 0.18ml of ascites, thus making the method economically unfeasible for our purpose.

To be feasible, cross-adsorption of the rabbit antisera will require a ferret sIgA immunoaffinity column for isolating sIgA-reactive antibodies. Passage of the antisera through this column and the ferret IgG column should yield an sIgA-specific product. To prepare a ferret sIgA-Affi-gel column, 10-20mg of purified sIgA will be necessary; however, the yield from our current ferret milk stock (~40 μ g/ml) makes purification of such amounts impractical. Our yields suggest that we have been using mature milk as a source of sIgA. Since colostrum should contain a significantly higher concentration of sIgA (100-200 fold), we contacted the Navy in October and inquired whether we had received mature milk or colostrum. The Navy researchers were unable to determine the identity of the material at that time; however, we later identified and contacted a company from which ferret colostrum was purchased for analysis.

As of this writing, we have received the ferret colostrum and have begun analysis of its sIgA content. During the current quarter, we will determine the sIgA concentration in the ferret colostrum. If the sIgA concentration in the colostrum is greater than that of the ferret milk provided by the Navy, we will purify some sIgA to determine a yield. If a sufficient yield is achieved, sIgA will be purified from the remaining colostrum and a ferret sIgA-Affi-prep matrix will be prepared. If the yield is inadequate, we will examine ferret serum to determine whether it can be used as a source of IgA.

2. Extraction and Purification of Lipopolysaccharide

We will complete the production and analysis of *C. jejuni* LPS, then deliver the material to the Navy.

4. Type-Specific Campylobacter Flagellin Epitopes

We will complete immunizing two mice, perform a splenocyte-myeloma fusion, and begin screening and characterizing MAbs produced by the resultant hybridomas.

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